

## METHOD FOR TREATMENT OF TUMORS USING PHOTODYNAMIC THERAPY

### FIELD OF THE INVENTION

The present invention relates to methods for treating tumors. In particular, the present invention involves treating tumor-bearing individuals with photodynamic therapy in combination with additional reagents.

### BACKGROUND OF THE INVENTION

Photodynamic therapy (PDT) is a treatment for cancer that involves the use of a photosensitizer and light. In this treatment modality, an individual afflicted with cancer or precancerous condition is administered a photosensitizing agent. Cancerous (and precancerous) cells retain the photosensitizer more readily than normal tissues. Subsequent exposure of the cells to wavelength-specific light induces a photochemical reaction that causes oxidative damage to numerous cellular components and cell death (reviewed by Dougherty et al., *J. Natl. Cancer Institute* 90:889; 1998).

CD40 is a transmembrane protein expressed on various normal cells, including B lymphocytes, monocytes some epithelial cells and dendritic cells, as well as on various transformed carcinoma cell lines (Clark, *Tissue Antigens*, 36:33 (1990). A ligand for CD40 is expressed on activated T cells (Spriggs et al, *J. Exp. Med.*, 176:1453 (1992); Armitage et al, *Nature*, 357:80 (1992). Binding of CD40 with CD40L causes B cell proliferation in the absence of any co-stimulus, and induction of antibody secretion from B cells in the presence of cytokines.

Soluble forms of CD40L and agonistic CD40 antibodies (i.e., those that mimic the biological effects of CD40L) are useful in the treatment of diseases characterized by neoplastic cells that express CD40, such as B lymphomas, melanomas and carcinomas (U.S. Patent 5,674,492). Soluble CD40L has also been used to promote the proliferation and/or differentiation of CD40-positive sarcoma cells, as a means of directly treating the malignancy or as an adjunct to chemotherapy, or to increase the immune response of an immunosuppressed individual, such as a subject suffering from malignancy (U.S. Patent 5,945,513). Moreover, soluble CD40L has been used to stimulate a T effector cell-mediated immune response (WO96/26735).

### SUMMARY OF THE INVENTION

An object of the present invention is to provide an improved method for treating tumor-bearing subjects using PDT. Another object of the present invention is to provide a method for treating tumor-bearing subjects, wherein cells of said tumor do not necessarily

express CD40. Yet another object of the present invention is to induce a memory CTL response in tumor-bearing subjects.

These and other objects of the present invention, which will be apparent for the detailed description of the present invention provided hereinafter, have been met, in one embodiment, by a method for treating a tumor-bearing subject comprising administering a therapeutically effective amount of a CD40 binding protein to said subject in combination with photodynamic therapy.

In another embodiment, the above-described objects of the present invention have been met by a method for treating a tumor-bearing subject comprising administering a therapeutically effective amount of a CD40 binding protein to said subject in combination with photodynamic therapy, wherein cells of said tumor do not express CD40.

In yet another embodiment, the above described objects of the present invention have been met by a method for inducing a memory cytotoxic T lymphocyte (CTL) response in a tumor-bearing subject comprising administering a therapeutically effective amount of a CD40 binding protein to said subject in combination with photodynamic therapy, wherein the memory CTL response is specific to the tumor.

The present invention further encompasses the above identified methods for treating tumor-bearing subjects and methods for inducing a CTL response that further include administering additional therapeutic or active agents. Such therapeutics or active agents include those that induce tumor cell death and/or apoptosis, those that increase the numbers of antigen-presenting cells, those that stimulate maturation of dendritic cells and those that lead to T effector cell expansion and immune activation. Suitable additional therapeutic or active agents include FasL, TRAIL, TNF alpha and CD30L.

The present invention further contemplates, in combination with the above identified methods, *in vivo* and/or *in vitro* methodologies that involve immune based tumor therapy and/or dendritic cell expansion and maturation techniques for optimizing anti-tumor therapeutic effects of PDT and CD40L. More particularly, the present invention includes methods for treating tumor-bearing individuals that involve administering Flt3L to the tumor bearing individual; administering photodynamic therapy to the individual, and administering CD40L to the individual. Additional therapeutic or active agents that induce tumor cell death and/or apoptosis, increase the numbers of antigen-presenting cells and stimulate dendritic cell maturation can be administered as well. The present invention further encompasses *in vitro* methodologies that involve collecting dendritic cells from the individual, expanding the dendritic cells by exposing them to Flt3-L, infusing the expanded dendritic cells into the individual, treating the individual with photodynamic therapy and administering CD40 binding protein to the individual. Prior to collecting the dendritic cells, administering flt3-L to the individual will aid in dendritic cell mobilization and increase the number of dendritic cells available for collection. Alternatively, *in vitro* methods can include collecting

hematopoietic stem or progenitor cells and contacting the cells with flt3-L to generate dendritic cells, prior to infusing the generated dendritic cells into the tumor bearing individual.

A variety of CD40 binding proteins may be employed in the present invention, including, for example, an antibody that binds CD40; full-length-membrane bound CD40L; a soluble extracellular region of a CD40L; a fusion protein comprising a CD40 binding region (or domain) from a CD40L or an antibody to CD40, fused to a second protein, for example, an immunoglobulin Fc domain or a zipper domain.

Suitable CD40 antibodies include CD40 antibodies that bind and crosslink CD40, thereby transducing a signal. Among these are monoclonal antibody HuCD40-M2 (ATCC HB11459) and CD40 binding proteins comprising an antigen-binding domain derived from antibody HuCD40M2.

### **DETAILED DESCRIPTION OF THE INVENTION**

It is believed that in the present invention, a tumor killing or lysing procedure known as Photodynamic therapy (PDT) induces cell death, and leads to antigen uptake and presentation by dendritic cells (DC) in sites draining the dying tumor. When contacted with a CD40 binding protein, these tumor antigen-bearing DC induce a potent memory CTL response specific to the tumor. The CTL response leads to eradication or significant reduction of the remaining tumor burden. The methods described herein can be used to treat a wide range of tumors and precancerous cells, including, but not limited to, basal and squamous cells, skin cancers, breast cancer, cancers that are metastatic to skin, brain tumors, head and neck, stomach, and female genital tract malignancy, cancers and precancerous conditions of the esophagus such as Barrett's esophagus.

The present invention encompasses combining PDT with administering CD40 binding protein in a tumor bearing subject. In another embodiment, the methods of the present invention further include combination therapies of administering one or more active agents for enhancing immune-based tumor therapy. More particularly, in addition to administering CD40 binding protein in combination with PDT, the present invention includes administering one or more mobilization agents for increasing dendritic cells numbers; and/or administering one or more agents for inducing dendritic cell maturation; and/or administering one or more agents which stimulate T cell proliferation.

Dendritic cells can be increased *in vivo* by administering Flt3L and/or GM-CSF to the tumor bearing subject. Suitable agents for inducing dendritic cell maturation include CD40L, TNF alpha, RANKL, LPS, and conditioned monocyte media. Dendritic cell maturation agents can be administered systemically or locally, at or near the tumor site. Suitable agents for stimulating T cell proliferation and function include, but are not limited to, IL-2, IL-15,

IL-7, IL-12, and IFN gamma. Agents that stimulate T cell proliferation may be administered systemically or in the vicinity of the tumor or the draining lymph nodes.

In addition to, or as an alternate to the *in vivo* methods for generating dendritic cells, dendritic cells can be generated using *in vitro* methods and subsequently administered to the tumor-bearing subject. For example, CD34+ cells can be collected, utilizing known collection and cell separation methods, subsequent to *in vivo* mobilization with Flt3L, G-CSF, GM-CSF, SCF or cyclophosphamide, and/or other mobilization agents. Dendritic cells from the collected CD34+ cells can be grown *in vitro* using dendritic cell generation active agents such as Flt3L, GM-CSF, CD40L, and IL-15. Alternatively, PBMC can be collected for the purpose of generating dendritic cells *in vitro*, optionally using reagents such as GM-CSF and IL-4 to generate the dendritic cells. The *in vitro* generated dendritic cells may be infused into the PDT receiving tumor-bearing subject in order to increase the number of dendritic cells for inducing a CTL response.

Methods for the *in vivo* and *in vitro* mobilization and generation of dendritic cells and methods for stimulating T cell proliferations are described in WO 97/12633 and copending U.S. applications S/N 09/154,903, 09/444,027, 09/448,378, all of which are incorporating herein by reference. The methods described in these references are suitable for use in the practice of the present invention.

#### Photodynamic Therapy

The tumor killing or lysing procedure utilized in the present invention, PDT, is a cancer treatment that utilizes a photochemical reaction to destroy neoplastic cells and cells that are pre-cancerous or precursors to neoplastic cells (reviewed by Dougherty et al., *J. Natl. Cancer Institute* 90:889; 1998). The application of PDT is according to methods known in the art which generally involve administering one or more photosensitizers to a tumor bearing subject followed by a light activation step in which light of a specific wavelength is directed to the tumor where the photosensitizer is lodged.

The cytotoxic effect of PDT is primarily mediated by the formation of singlet oxygen generated by energy transfer from a light-activated, tissue localized photosensitizer to ground state oxygen. Singlet oxygen has a short radius of action, and can cause oxidative damage to numerous cellular components at or near the site of its generation (Gollnick et al, *Cancer Res.*, 57:3904-3909 (1997)).

The oxidative damage mediated by PDT has a variety of effects on tumor cells, the microvasculature within and near the tumor, and on cells of the immune system. PDT induces changes in the plasma membrane and membranes of cellular organelles of affected cells, upregulating expression of some stress protein genes and activating certain genes involved in apoptosis, as well as leading to the release of powerful inflammatory mediators. Although the role of the various effects induced by PDT is not clear, it is believed that the

combination of the effects is necessary for eradication of cancer cells (Dougherty et al., *supra*).

The acute phase immune reaction to PDT is inflammatory in nature; control of tumors over the long term, however, appears to be a result of specific anti-tumor immunity. The effectiveness of such long-term, tumor-specific immunity is unpredictable because PDT can significantly suppress certain immune functions, especially those involving effector T cells (Elmets et al, *Cancer Res.*, 46:1608-1611 (1986); Simkin et al, *Proc. Int. Soc. Optical Eng.*, 2392:2333 (1995); Gruner et al, *Scand. J. Immunol.*, 21:267-273 (1985)). The suppressive effects are believed to involve mediation of immune-modulating cytokines, such as IL-6 and IL-10 (Gollnick et al, *supra*).

PDT has been used effectively in the treatment of a variety of human tumors and precancerous conditions, including basal and squamous cells, skin cancers, breast cancer, metastatic to skin, brain tumors, head and neck, stomach, and female genital tract malignancy, cancers and precancerous conditions of the esophagus such as Barrett's esophagus (U.S. Patent 6,013,053, which is incorporated by reference herein in its entirety; Marcus, In: *Future Directions and Applications in Photodynamic Therapy*, Gomer, Ed., Bellingham, WA SPIE Optical Engineering Press (1990) pages 5-56; and Overholt et al, *Sem. Surg. Oncol.*, 11:1-5 (1995)).

Examples of useful photosensitizer which can be employed in the present invention include hematoporphyrins (Kessel, *Cancer Lett.*, 39:193-198 (1988), uroporphyrins, phthalocyanines (Kreimer-Birnbaum, *Sem. Hematol.*, 26:157-173 (1989), purpurins (Morgan et al, *Photochem. Photobiol.*, 51:589-592 (1990); and Kessel, *Photochem. Photobiol.* 50:169-174 (1989), acridine dyes, bacteriochlorophylls (Beems et al, *Photochem. Photobiol.*, 46:639-643 (1987); and Kessel et al, *Photochem. Photobiol.*, 49:157-160 (1989), and bacteriochlorins (Gurinovitch et al, *J. Photochem. Photobiol. B-Biol.*, 13:51-57 (1992)).

Photosensitizers suitable for use in the present invention include those summarized, in part, in Table 1 of U.S. Patent 5,942,534, which is incorporated by reference herein in its entirety. An alternative to administration of the photosensitizer itself, is administration of a precursor of that compound. For example, 5-aminolevulinic acid causes endogenous production of the photosensitizer protoporphyrin IX (Morgan et al, *J. Med. Chem.*, 32:904-908 (1989).

#### CD40/CD40 Binding Proteins

CD40 is a member of the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family that has been found to be expressed on B lymphocytes, monocytes, some epithelial cells and dendritic cells (Clark, *Tissue Antigens*, 36:33; 1990). This cell surface antigen has been shown to play an important role in B cell proliferation and differentiation, and in the growth of malignant cells upon which it is expressed.

The ligand for CD40 (hereinafter "CD40L") has been identified and characterized, and DNA encoding the same has been cloned from peripheral blood T cells (Spriggs et al, *J. Exp. Med.*, 176:1453 (1992); Armitage et al, *Nature*, 357:80 (1992); and Armitage et al, U.S. Patent Nos. 5,961,974, 5,962,406 and 5,981,724; each of which is incorporated by reference  
5 herein in its entirety). CD40L biological activity is mediated by binding of this cytokine with CD40, and includes B cell proliferation in the absence of any co-stimulus, and induction of antibody secretion from B cells, in the presence of cytokines.

As used herein, "CD40 binding protein" refers to polypeptides that specifically bind CD40 in a noncovalent interaction based upon the proper conformation of the CD40 binding  
10 protein and CD40 itself. Preferably, the CD40 binding protein has agonistic activity, that is, it mimics the native ligand for CD40 (CD40L) that is present on activated T cells by binding to, and transducing a signal to, a cell expressing CD40. Assays for biological activities of CD40L are useful for assessing agonistic activity. Additional methods to measure agonistic activity of a CD40 binding protein include analyzing CD40 binding protein for the ability to  
15 inhibit binding of CD40 to CD40L. CD40 binding proteins that bind CD40 and inhibit binding of CD40 to CD40L, as determined by observing at least about 90% inhibition of the binding of soluble CD40 to CD40L, will have agonistic activity.

The CD40 binding proteins useful in the present invention include antibodies to CD40 (including humanized antibodies or antibodies that have been manipulated through recombinant means to render them suitable for therapeutic use), CD40L, soluble CD40L, and  
20 fusion proteins comprising a soluble CD40L or an antibody to CD40, and a second protein. More particularly, CD40 binding proteins include antibodies to CD40 that crosslink CD40 and transduce a signal; full-length CD40L; oligomeric soluble forms of CD40L or fragments thereof that bind CD40 (e.g. the CD40L extracellular domain and fragments thereof); CD40L  
25 fusion proteins, e.g. soluble CD40L/Fc fusions and soluble CD40L/leucine zipper fusions. Oligomeric soluble forms of CD40L include the extracellular domain of CD40L or fragments of the extracellular domain that bind CD40 that are in oligomeric form. One such example of soluble oligomeric CD40L is the extracellular domain fragment of amino acids 113-261 of SEQ ID NO:2 and the leucine zipper of SEQ ID NO:3. When the fragment and the leucine  
30 zipper are combined, an oligomeric form of CD40L results.

Full length CD40L includes polypeptides comprising amino acids 1 through 260 of SEQ ID NO:1 and amino acids 1 through 261 of SEQ ID NO:2. Soluble forms of CD40L include amino acids 47 through 260, 113 through 260, and 120 through 260 of  
35 SEQ ID NO:1 and amino acids 47 through 261, 112 through 261, 113 through 261, and 120 through 261 of SEQ ID NO:2. Further, CD40 binding proteins include fragments of the extracellular domain of CD40L (SEQ ID NO:1 and SEQ ID NO:2) that bind CD40. Such binding is sufficient to inhibit binding of soluble CD40 to CD40L, as determined by observing at least about 90% inhibition of the binding of soluble CD40 to CD40L.

Alternative embodiments of CD40L polypeptide, soluble CD40L polypeptides and suitable fragments thereof include polypeptides in which a cysteine at amino acid 194 of SEQ ID NO:2 is substituted with tryptophan. Still additional embodiments are encompassed by CD40L polypeptide and soluble CD40L polypeptides that are encoded  
5 by the complement of DNA that hybridizes to a DNA encoding any of the aforementioned polypeptides under conditions of severe stringency (hybridization in 6 X SSC at 63°C overnight; washing in 3 X SSC at 55°C) and which binds soluble CD40. Such binding is sufficient to inhibit binding of soluble CD40 to CD40L, as determined by observing at least about 90% inhibition of the binding of soluble CD40 to CD40L.

10 A preferred CD40 binding protein is an oligomeric soluble CD40L in which the soluble portion is an oligomerized extracellular domain fragment of SEQ ID NO:2 and the cysteine at amino acid 194 is substituted with tryptophan. Preferably, the oligomeric soluble CD40L includes an oligomerizing zipper domain (e.g. leucine zipper) such as that of SEQ ID NO:3 or a variant peptide in which conservative amino acid substitutions have been made,  
15 wherein the peptide is capable of forming an oligomeric soluble CD40L fusion protein. One such soluble oligomeric CD40L/leucine zipper fusion protein includes a polypeptide having amino acids 113-261 of SEQ ID NO:2 and the leucine zipper of SEQ ID NO:3 (CD40L/LZ).

Methods for expression of recombinant CD40L polypeptides are also described in the Armitage patents. Similar methods may be used for expression of other CD40 binding  
20 proteins. Moreover, numerous expression systems are known to those of routine skill in the art of molecular biology, including prokaryotic and eukaryotic expression systems. The expression system selected may affect the nature of the recombinant CD40 binding protein expressed. For example, CD40L expressed in mammalian expression systems (e.g., COS7 cells) may be similar to a native CD40L in molecular weight and glycosylation pattern,  
25 whereas CD40L expressed in yeast may be more highly glycosylated than native CD40L. Expression of CD40L in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

Antibodies to CD40 which can be employed in the present invention may be polyclonal or monoclonal. The particular agonistic CD40 antibody employed in the present  
30 invention is not critical thereto. Examples of such CD40 antibodies include HuCD40-M2 (ATCC No. HB11459) and HuCD40-M3, and antigen binding domains thereof. Additional CD40 mAbs which can be employed in the present invention may be generated using conventional techniques (see U.S. Patents RE 32,011, 4,902,614, 4,543,439, and 4,411,993, which are incorporated by reference herein in their entirety. Useful agonistic antibodies may  
35 also be constructed utilizing recombinant DNA techniques to "humanize" a murine antibody, or prepare single-chain antibodies, as described in U.S. Patent 5,801,227.

Once suitable CD40 binding proteins have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art. Suitable

techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques. Recombinant CD40 binding proteins can be prepared according to standard methods, and tested for binding specificity to the CD40 utilizing assays known in the art, including for example ELISA, ABC, or dot blot assays, as well by bioactivity assays such as those described for CD40 mAb.

#### Administration of CD40 Binding Protein

The CD40 binding protein may be administered in a suitable diluent or carrier to a subject, preferably a human. Thus, for example, CD40 binding protein can be given by bolus injection, subcutaneous or IP, continuous infusion, intermittent IV infusion, sustained release from implants, or other suitable technique.

Typically, a CD40 binding protein will be administered in the form of a pharmaceutical composition comprising purified CD40 binding protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers are nontoxic to subjects at the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining a CD40 binding protein with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents.

The particular therapeutically effective amount employed is not critical to the present invention, and will vary depending upon the particular CD40 binding protein selected, the type, frequency and intensity of PDT, as well as the age, weight and sex of the subject. Typically, therapeutically effective dosages, (doses that provide anti-neoplastic activity or doses sufficient to provide an enhanced CTL response) of CD40 binding proteins will be in the range of from about 0.01 to about 1.0 mg/kg body weight. More typically doses are in the range of 0.05 to 0.2 mg/kg bodyweight. As described below, administering CD40 binding protein can be carried out one or more days prior to administering PDT, continuing for a period of time in which the enhanced CTL response and enhanced immune response and/or enhanced antigen presenting cell maturation is effective. Alternatively administering CD40 binding protein can commence the day of or days following PDT. In any case, it is preferred that CD40 binding protein be present to enhance an immune response concurrent with or immediately following PDT.

CD40 binding proteins may also be used in conjugates of, or combination with, drugs, toxins or radioactive compounds. Preparation of such conjugates for treatment of various diseases are known in the art (see, for example, Waldmann, *Science*, 252:1657 (1991)).

### Administration of PDT

Photodynamic therapy, or PDT, is carried out by methods known in the art. Methods for administering PDT are described in Dougherty et al., *J. Natl. Cancer Institute* 90:889; 1998, incorporated herein by reference. Such methods include administering a photosensitizer or a mixture of photosensitizers, followed by exposure of the subject (the affected body area) to light that is absorbed by the photosensitizer. Subsequent to absorbing the light, the photosensitizer becomes excited and causes the generation of singlet oxygen. Singlet oxygen is highly toxic, but has a short radius of action. Various modes of administering a photosensitizer are known in the art, and will be useful in the present invention. For example, the photosensitizer may be administered orally, topically, parenterally, or locally (i.e., directly into or near the tumor or precancerous area). The photosensitizers may also be delivered using vehicles such as phospholipid vesicles or oil emulsions. Use of lipid-based delivery vehicles may result in enhanced accumulation of the photosensitizer in neoplastic cells. Alternative methods of delivery also encompassed in the instant invention include the use of microspheres, or monoclonal antibodies or other proteins that specifically bind a protein (or proteins) located on the surface of neoplastic cells.

The particular photosensitizer employed is not crucial to the present invention. Examples of photosensitizers useful in the present invention include hematoporphyrins, uroporphyrins, phthalocyanines, purpurins, acridine dyes, bacteriochlorophylls, bacteriochlorins and others are disclosed herein. A preferred photosensitizer employed is Photofrin® (QLT, Vancouver, Canada); additional examples are disclosed herein, and discussed in Dougherty et al. as well as various other resources disclosed herein.

The amount of photosensitizer administered will vary depending upon the particular photosensitizer employed, the age, weight and sex of the subject, the mode of administration, as well as the type, size and location of the tumor. For example, Photofrin® can be used at doses of 2.0 or 2.5 mg per kg body weight. The dosing for other types of photosensitizers can vary, ranging from 0.3 to 7.2 mg per kg body weight. Accordingly, those of skill in the art are able to determine preferred doses of various photosensitizing agents after examination of the relevant dosing information from the manufacturer and/or other experts in the field.

The wavelength of light to which the subject is exposed will vary depending upon the photosensitizer employed, and the location and depth of the tumor or precancerous cells. Generally, the subject will be exposed to light having a wavelength of about 600 to 900 nm, preferably about 600 to about 640 nm for Photofrin®. Several other photosensitizing agents have stronger absorbances at higher wavelengths, from about 650 to 850 nm, which can be beneficial for deeper tumors because light of longer wavelength tends to penetrate further into tissue. Conversely, a wavelength of about 410 nm may give better results when shallow penetration is desired; such dosages also fall within the scope of this invention.

The dose of light to which the subject is exposed will vary depending upon the photosensitizer employed. Generally, the subject will be exposed to light dose of about 50 to 500 J/cm<sup>2</sup> of red light, for Photofrin®. Other sensitizers may be more efficient, and thereby require smaller fluences, typically about 10 J/cm<sup>2</sup>. At higher fluences, hyperthermia may occur, which can enhance PDT; moreover, hyperthermia and PDT may act synergistically. Several different light sources are known in the art; any suitable light source capable of delivering an appropriate dosage of a selected wavelength may be used in the inventive methods.

The timing of light exposure will depend on the photosensitizer used, the nature and location of the tumor or precancerous cells, and the methods of administration. Typically, light exposure occurs at about one hour to four days after administration of the photosensitizer. Moreover, shorter time periods may be used, again depending on the photosensitizer, and the nature and location of the tumor. For example, light exposure after topical administration of a photosensitizer may occur as early as about ten minutes, or at about three hours after administration (see U.S. Patent 6,011,563, which is incorporated by reference herein in its entirety).

#### Enhancing Immune-based Tumor Therapy with Combination Therapies

The methods of the present invention further include administering one or more active agents for enhancing immune-based tumor therapy. More particularly, in addition to administering CD40 binding protein in combination with PDT, the present invention includes administering one or more mobilization agents for increasing dendritic cell numbers; and/or administering one or more agents for inducing dendritic cell maturation; and/or administering one or more agents which stimulate T cell proliferation, T effector cell expansion and immune activation.

Dendritic cells can be increased *in vivo* by administering Flt3L (described in U.S. Patent No. 5,554,512) and/or GM-CSF to the tumor-bearing subject. For example, prior to administering PDT to a tumor bearing individual, Flt3-L can be administered for a period of between about 2 days to 18 days and preferable for from 10 to 14 days at a dose of 5 µg/kg to 250 µg/kg and preferably from 25 µg/kg to 150 µg/kg per day. Alternatively, Flt3L can be administered at levels ranging from 50 µg/kg to 450 µg/kg every 5 days.

In addition to, or as an alternate to *in vivo* methods for generating dendritic cells, dendritic cells can be generated using *in vitro* methods and subsequently administered to the tumor-bearing subject. For example, prior to administering PDT and subsequent to using *in vivo* mobilization with Flt3L, G-CSF, GM-CSF, cyclophosphamide, SCF and/or other mobilization agents, CD34+ cells, stem or progenitor cells can be collected utilizing known collection and cell separation. Dendritic cells from the collected CD34+, stem or progenitor cells can be grown *in vitro* using dendritic cell generation active agents such as Flt3L, GM-

CSF, CD40L or other CD40 binding protein, and IL-15. Alternatively, PBMC can be collected for the purpose of generating dendritic cells *in vitro*. The *in vitro* generated dendritic cells may be infused into the PDT receiving tumor-bearing subject in order to increase the number of dendritic cells for inducing a CTL response. Cell culture media that incorporate Flt3-L and/or other agents for the *in vitro* generation and mobilization of dendritic cells include these agents in quantities sufficient to maximize the number of dendritic cells for the later infusion into the tumor-bearing subject or precancerous bearing subject. Such amounts may range from 0.1 µg/mL to 5 µg/mL and typically are about 2 µg/mL.

Methods for the *in vivo* and *in vitro* mobilization and generation of dendritic cells and methods for stimulating T cell proliferations are described in WO 97/12633 and copending U.S. applications S/N 09/154,903, 09/444,027, 09/448,378, all of which are incorporated herein by reference. The methods described in these references are suitable for the practice of the present invention.

In accordance with the present invention CD40 binding proteins may be administered to stimulate maturation of DC, enhancing their capabilities to stimulate an effective, specific, anti-tumor cytotoxic response. CD40 binding proteins may be used in conjunction with other DC-maturation factors, such as TNF-alpha, a ligand for the receptor activator of NF-kappaB (RANKL), and substances such as lipopolysaccharide. Moreover, agents that enhance a CTL response may be use in conjunction with a CD40 binding protein. Such agents include Interleukins 2, 15, 7 and 12, and interferons-gamma and -alpha. Dendritic cell maturation agents can be administered systemically or locally, at or near the tumor site. Doses of CD40 binding proteins and specifically oligomeric soluble forms of CD40L, can range from 0.01 mg/kg to 1 mg/kg, and are preferably in the range of 0.05 mg/kg to 0.2 mg/kg. Dosing frequency can range from every day, to every other day and may be limited to once per week when the mode of administration favors such frequency (e.g. by i.v. administration).

Use of a CD40 binding protein in conjunction with PDT, in accordance with the present invention, means that the CD40 binding protein may be administered before, during or after PDT. Preferably, a CD40 binding protein is administered after PDT, most preferably CD40 binding protein administration begins on the day of, or about one to two days after PDT administration. Furthermore, the combination of a CD40 binding protein and PDT may be supplemented by the use of additional active agents as described herein. Additional active agents may be administered at the same time as, before, or after, administration of CD40 binding proteins, as appropriate for the agent and desired result. For example, FasL, TRAIL, CD30L and TNF alpha may be administered concurrent with administering CD40 binding protein. The presence of these active agents in combination therapies enhances the tumor eradicating characteristics of the combination of CD40 binding protein and PDT. In one

embodiment the active agent or active agents are administered intra-tumor or close to the tumor.

Because PDT is an entirely different process from radiotherapy (ionizing radiation), chemotherapy and surgery, and thus the use of PDT is not precluded by prior radiotherapy, chemotherapy or surgery (Hsi et al, *Drugs*, 57:7250734 (1999); and McCaughan, *Drugs & Aging*, 15:49068 (1999)), it can be used in conjunction with such processes (i.e., before, during or after an alternative process such as radiation therapy). The relatively low toxicity of PDT also makes it suitable as a repeatable form of therapy. Furthermore, the improvements described herein may render it possible to further reduce side effects, by decreasing the amount of photosensitizer or the dosage of light needed.

#### Prevention or Treatment of Disease

These results presented herein indicate that CD40 binding proteins may be of significant clinical use in the treatment of various tumors. The term treatment, as it is generally understood in the art, refers to initiation of therapy after clinical symptoms or signs of disease have been observed. In one embodiment, the tumor may express CD40, for example, B lymphomas, melanomas or sarcomas. In another embodiment, the tumor does not express CD40. Examples of such tumors include T cell lymphomas and leukemias, many connective tissue tumors, and neuroblastomas.

Furthermore, the present invention will be useful in the treatment of precancerous conditions (such as Barrett's esophagus) for which PDT can be employed. When employed in this manner, the inventive methods described herein may be thought of as preventative measures rather than strictly defined treatment of an afflicted individual.

The present invention may be used in conjunction with other therapies appropriate for afflicted subjects, including chemotherapy, radiation therapy, and immunotherapy.

The relevant disclosures of all references cited herein are specifically incorporated by reference. The following examples are intended to illustrate particular embodiments, and not limit the scope, of the invention. Those of ordinary skill in the art will readily recognize that additional embodiment are encompassed by the invention.

#### EXAMPLE 1

This example demonstrates that the protective antitumor response induced by PDT *in vivo* is dependent on the CD40:CD40L interaction. Female BALB/c mice (n=45) were inoculated subcutaneously (SQ) with  $5.0 \times 10^4$  BALB/c mouse mammary carcinoma EMT6 cells. On day 6, post tumor inoculation, 30 of the tumor-bearing mice were injected intraperitoneally (IP) with Photofrin® (a photosensitizer obtained from QLT, Vancouver, Canada) at a dose of 5.0 mg/kg. The remaining 15 tumor-bearing mice were not given

Photofrin (negative control). The following day (day 7), 15 of the Photofrin-injected tumor-bearing mice were treated with  $135 \text{ J/cm}^2$  of red light having a wavelength of 630 nm. Tumors from the remaining 15 Photofrin-injected tumor-bearing mice, which were not exposed to the red light, were surgically removed. Immediately following light treatment or surgery, 5 mice of each treatment group received IP, 200  $\mu\text{g}$  of rat IgG (Sigma), 200  $\mu\text{g}$  of rat monoclonal anti-muCD40L M158 (Immunex Corporation, Seattle, WA), or no antibody injection. These antibody injections were repeated on days 8, 10 and 12. The negative control mice group was similarly injected with antibodies on days 7, 8, 10 and 12. On day 13, lymph node cells from all of the treatment groups were isolated and mixed with fresh EMT6 tumors cells at a ratio of 500 lymph node cells to 1 tumor cell, i.e.  $2.5 \times 10^6$  lymph node cells and  $5.0 \times 10^4$  EMT6 cells, and the resulting mixture was injected SQ into non-tumor bearing BALB/c mice. Tumor incidence and tumor growth was monitored from day 18 to day 90. The results are shown in Table 1 below.

Table 1: Role of CD40/CD40L Interaction in the  
Protective Anti-tumor Response Induced by PDT  
Tumor take (%incidence)

Tumor Therapy	Antibody Treatment	at day 90
None	None	5/5 (100%)
None	Rat IgG	4/5 (80%)
None	Anti-CD40L M158	5/5 (100%)
PDT	None	1/5 (20%)
PDT	Rat IgG	0/5 (0%)
PDT	Anti-CD40L M158	3/5 (60%)
Surgical removal	None	4/5 (80%)
Surgical removal	Rat IgG	5/5 (100%)
Surgical removal	Anti-CD40L M158	4/5 (80%)

As shown in Table 1, treatment of tumor-bearing mice with PDT induced a strong anti-tumor immune response that was transferred to naive mice challenged with the EMT6 tumor; 9/10 or 90% of mice that received PDT therapy developed a protective anti-tumor immune response. However, administration of M158, a muCD40L specific antibody that neutralizes CD40L biological activity, to mice after PDT therapy prevented the development of a protective anti-tumor immune response in 60% of mice. The control rat IgG protein did not alter the development of PDT-induced anti-tumor immunity. Surgical removal of the tumor did not induce a protective immune response either.

These findings indicate that CD40L function is required for development of a protective anti-tumor immune response that occurs following PDT treatment. That CD40L is

biologically important in the generation of PDT-induced protective anti-tumor immunity *in vivo*, is a significant and novel observation. Since PDT is a unique treatment, it can be used when surgery, chemotherapy and/or radiation have not eliminated the cancer. Combining PDT with administration of a CD40 binding protein should significantly enhance the *in vivo* anti-tumor immune response to a variety of tumors.

### **EXAMPLE 2**

This example demonstrates that the administration of a CD40 binding protein (soluble, trimeric CD40L referred to as CD40LT) to tumor-bearing subjects in conjunction with PDT *in vivo* enhances anti-tumor treatment. Female BALB/c mice are inoculated subcutaneously (SQ) with tumor cells derived from a weakly immunogenic tumor. The tumor cells are allowed to grow for a time sufficient to establish a tumor that cannot be totally eradicated with PDT; a portion of the mice are injected intraperitoneally (IP) with Photofrin® (a photosensitizer obtained from QLT, Vancouver, Canada) at a dose of 5.0 mg/kg. The remaining tumor-bearing mice are not given Photofrin® (negative control). The day following Photofrin® administration, half of the Photofrin-injected, tumor-bearing mice are treated with 135 J/cm<sup>2</sup> of red light having a wavelength of 630 nm. Tumors from the remaining Photofrin-injected, tumor-bearing mice, which were not exposed to the red light, are surgically removed. Within one to two days following light treatment or surgery, half of the mice in each treatment group receive, IP, 200 µg of rat IgG (Sigma), or CD40LT. Tumor incidence and tumor growth is monitored as needed. The table below presents a treatment matrix used to allocate an appropriate number of mice to each group.

Table 2: Evaluation of CD40LT/PDT Combination Therapy

<u>Tumor Therapy</u>	<u>Antibody Treatment</u>
None	Rat IgG
None	CD40LT
PDT	Rat IgG
PDT	CD40LT
Surgical removal	Rat IgG
Surgical removal	CD40LT